

Increased levels of nitric oxide derivatives in induced sputum in patients with asthma

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Background: Nitric oxide (NO) plays an important role as an inflammatory mediator in the airways. However, because direct measurement of endogenous NO has been difficult in vivo, the exact pathologic roles of NO in human airway inflammation have remained unclear.

Objective: This study was designed to determine whether NO may be harmful by amplifying allergic inflammation in asthmatic airways.

Methods: In this study we examined the concentration of stable end products of NO, nitrite and nitrate, in induced sputum in 18 patients with asthma and 10 normal control subjects and evaluated the relationship between levels of NO derivatives in sputum and cellular and biochemical profiles, the degree of airflow obstruction, and the cytotoxic activities for epithelial cells.

Results: The concentration of NO derivatives in induced sputum was significantly higher in patients with asthma than in normal control subjects ($1086 \pm 325 \mu\text{mol/L}$, $577 \pm 115 \mu\text{mol/L}$; $p < 0.05$). Percentages of eosinophil counts and eosinophil cationic protein levels in sputum were also significantly higher in patients with asthma. Moreover, percentages of eosinophil counts and eosinophil cationic protein levels in sputum in patients with asthma were significantly correlated with the concentration of NO derivatives in sputum ($r = 0.63$, $p < 0.01$; $r = 0.56$, $p < 0.05$, respectively). In addition, we found that the concentration of NO derivatives in sputum in patients with asthma was significantly correlated with the degree of airflow obstruction ($\text{FEV}_1/\text{forced vital capacity}$) ($r = -0.62$, $p < 0.01$) and with percentages of shedding epithelial cells ($r = 0.61$, $p < 0.01$).

Conclusion: We have shown that a higher concentration of NO derivatives was found in induced sputum of patients with asthma as compared with normal subjects. The clinical implication of our findings is that measurement of NO derivatives in induced sputum may be useful for assessing allergic inflammation in airways. (*J Allergy Clin Immunol* 1997;99:624-9.)

Key words: Nitric oxide, induced sputum, bronchial asthma, eosinophil cationic protein, epithelial shedding

There is increasing evidence that endogenous nitric oxide (NO) plays an important role in physiologic regulation of the airways and is implicated in the pathophysiology of airway disease.¹ Nitric oxide is formed by the

Abbreviations used

cNOS:	Constitutive nitric oxide synthase
ECP:	Eosinophil cationic protein
ELF:	Epithelial lining fluids
FVC:	Forced vital capacity
iNOS:	Inducible nitric oxide synthase
NO:	Nitric oxide

enzyme NO synthase (NOS) from the semi-essential amino acid L-arginine, and at least three isoforms of this enzyme have been identified in human airways.^{2,3} Constitutive isoforms of NOS (cNOS) are expressed in endothelial cells and mediate endothelium-dependent vasodilatation, whereas another constitutive enzyme is present in nerves and is involved in the release of NO as a bronchodilator neurotransmitter in airways.⁴ A distinct isoform of NOS is inducible by proinflammatory cytokines NOS (iNOS) in several types of cells, including inflammatory cells and epithelial cells.^{5,6} There is an increasing interest in the possibility that endogenous NO may increase airway inflammation. Endogenous NO appears to directly exacerbate neurogenic plasma exudation in the airways.⁷ NO is a potent vasodilator in the bronchial circulation⁸ and may increase plasma exudation by increasing the blood flow to postcapillary venules made leaky by exposure to inflammatory mediators. In many pathologic conditions, including inflammation, simultaneous cellular production of superoxide anion and NO may occur, potentially leading to the formation of peroxynitrite, which may be an important mediator of free radical-induced cellular injury.^{9,10} We therefore hypothesized that NO plays an important role in the initiation and propagation of airway inflammation.

However, because direct measurement of endogenous NO has been difficult in vivo, the exact pathologic roles of NO in human airway inflammation have remained unclear. In this study we examined the concentration of stable end products of NO, nitrite and nitrate, in induced sputum and determined that NO may be harmful by amplifying allergic inflammation in patients with asthma.

METHODS

Subjects

A total of 18 patients with asthma (mean age, 36.2 years) and 10 normal control subjects (mean age, 34.4 years) volunteered for this study (Table I). Two of the patients were ex-smokers who had not smoked for more than 5 years, and the others were

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TABLE 1. Characteristics of patients with asthma

Patient No.	Age (yr)	Sex	IgE (IU/ml)	FEV ₁ (% pred)	PC ₂₀ methacholine (μg/ml)	Regular medication
1	30	M	103	111.8	2500	Oβ, THEO
2	26	M	379	72.8	1250	Oβ, Iβ, THEO
3	26	M	130	93.2	313	Oβ, Iβ
4	35	M	138	113.5	2500	Oβ, Iβ, THEO
5	25	F	168	123.8	2500	Oβ, Iβ
6	65	M	349	105.2	5000	Iβ
7	50	F	89	96.7	625	Oβ, Iβ, IS, THEO
8	64	M	249	98.6	2500	Oβ, Iβ, IS, THEO
9	52	M	223	152.5	5000	Oβ, Iβ, THEO
10	28	M	179	73.9	625	Oβ, Iβ, IS
11	29	F	1846	109.2	5000	Iβ, IS, THEO
12	28	M	150	108.0	625	Oβ, Iβ, THEO
13	25	M	451	127.8	1250	Iβ, THEO
14	25	M	5880	80.9	39	Oβ, THEO
15	40	F	379	75.9	313	Oβ, THEO
16	64	M	130	121.4	5000	Oβ, Iβ, THEO
17	20	F	238	85.8	625	None
18	20	F	185	108.2	5000	Iβ, THEO

Oβ, Oral β-agonist; Iβ, inhaled β-agonist used as needed; IS, inhaled steroid; THEO, oral theophylline.

nonsmokers who had never smoked. Patients with asthma had a history of wheezing or chest tightness and had been given a diagnosis of asthma by a physician. All patients had well-controlled symptoms, and four of the patients used inhaled corticosteroids. All had FEV₁ values greater than 70% of predicted normal value and bronchial hyperreactivity to methacholine. There was no history of upper respiratory tract infection for at least 4 weeks before the study, and none of the subjects had received antibiotics in the week preceding the study. Patients did not consume any caffeine for 2 hours or receive any medication for 18 hours before the study. All subjects gave their written informed consent for participation in the study, which was approved by the Ethics Committee of Osaka City University.

Sputum induction and processing

Spirometry was performed before inhalation of 200 mg of salbutamol through a metered-dose inhaler. All subjects were instructed to wash their mouths thoroughly with water. Subjects inhaled 3% saline solution at room temperature, nebulized by an ultrasonic nebulizer (NE-U12, OMRON Co., Tokyo, Japan) at maximum output. Subjects were encouraged to cough deeply after 5 minutes and at 3-minute intervals thereafter. Sputum was coughed into polypropylene containers, and one portion of the specimen was collected separately for total cell counting and slide making. After sputum induction, spirometry was repeated. If the FEV₁ had fallen, the subject was required to wait until it had returned to baseline value. The sputum samples were kept at 4° C for not more than 2 hours before further processing. The volumes of samples were recorded. The portion of the sample for cell counting was diluted with phosphate-buffered saline containing 10 mmol/L dithiothreitol (Sigma Chemical Co., St. Louis, Mo.) and gently vortexed at room temperature. The portions for cell counting were then centrifuged at 400 g for 10 minutes, and the cell pellet was resuspended. Total cell counts were performed with a hemocytometer, and slides were made by using a cytospin (Cytospin 3; Shandon, Tokyo, Japan) and stained with May-Grünwald-Giemsa stain for differential cell counts.

The supernatant was stored at -70° C for subsequent assay for eosinophil cationic protein (ECP) and albumin concentration. ECP concentration was measured by using a radioimmunoassay kit (Pharmacia Diagnostics, Uppsala, Sweden), and albumin concentration was measured by laser nephelometry.

Nitric oxide assay

Nitric oxide derivatives (nitrate + nitrite) were assayed colorimetrically after the Griess reaction as previously described.¹¹ Two hundred microliters of sputum sample or standard was deproteinized by adding 20 μl of NaOH (1.0 mol/L, 4° C; Wako Chemical Co., Osaka, Japan) and 30 μl of ZnSO₄ (1.3 mol/L, 4° C; Wako Chemical Co.). Samples were mixed and allowed to stand on ice for 15 minutes. After centrifugation (5 minutes, 4° C, 2600 g), 100 μl of the supernatant was mixed with 5 × 10⁻² units of nitrate reductase (Sigma Chemical Co.), 20 μl of 0.2 mol/L N-tris(hydroxymethyl)methylaminoethanesulfonic acid (pH 7.0, Sigma Chemical Co.), and 20 μl of 0.5 mol/L sodium formate (Wako Chemical Co.). After anaerobic incubation at room temperature for 20 minutes, 1.0 ml of water was added to the samples, and nitrite was assayed in supernatants obtained by centrifugation (5 minutes, 260 g). Deproteinized samples or standards (200 μl) were mixed with 20 μl of 1% sulfanilamide (Sigma Chemical Co.) in 15% phosphoric acid (Wako Chemical Co.). After 10 minutes, 20 μl of 0.1% N-(1-naphthyl) ethylenediamine (Sigma Chemical Co.) was added, and the absorption at 540 nm was determined.

Statistical analysis

Values are expressed as means ± SD. Statistical analysis of comparisons between two groups were performed by using the Mann-Whitney U test, and Spearman's rank-correlation coefficients were determined for analysis of correlation between variables. A *p* value of less than 0.05 was considered significant.

RESULTS

All subjects tolerated the sputum induction procedure well. There was no significant decrease in FEV₁ after

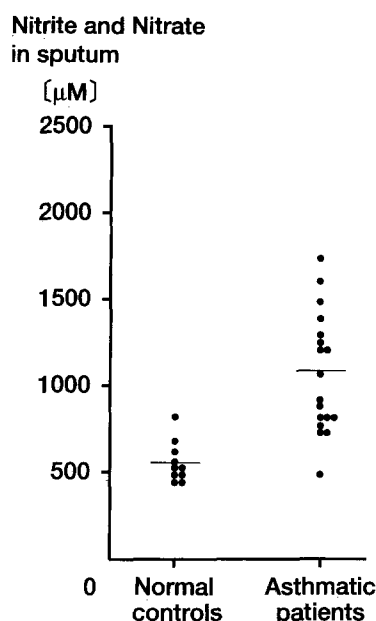


FIG. 1. Concentration of NO in induced sputum in patients with asthma and normal control subjects. Bars represent mean value. Concentration of NO in induced sputum in patients with asthma was significantly higher than that in induced sputum of normal control subjects ($p < 0.05$).

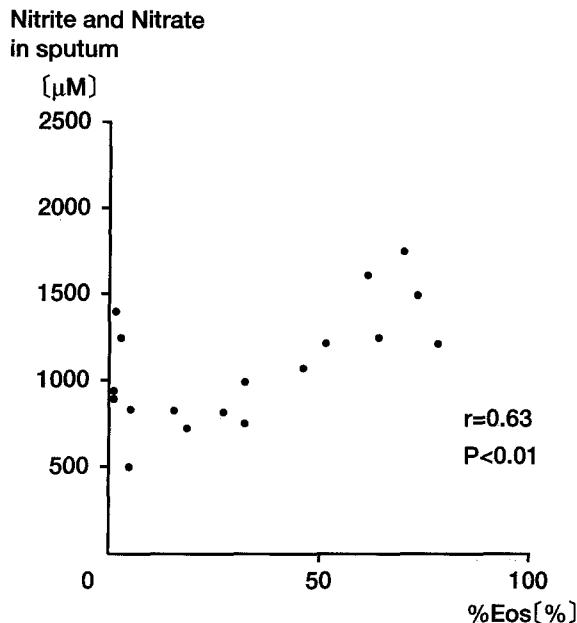


FIG. 2. Relationship between percentages of eosinophil counts and NO levels in induced sputum in patients with asthma. Eos, Eosinophils.

sputum induction in any subject, and no subject experienced shortness of breath or chest tightness.

The concentration of NO derivatives in induced sputum was significantly higher in patients with asthma than in normal control subjects ($1086 \pm 325 \mu\text{mol/L}$, $577 \pm$

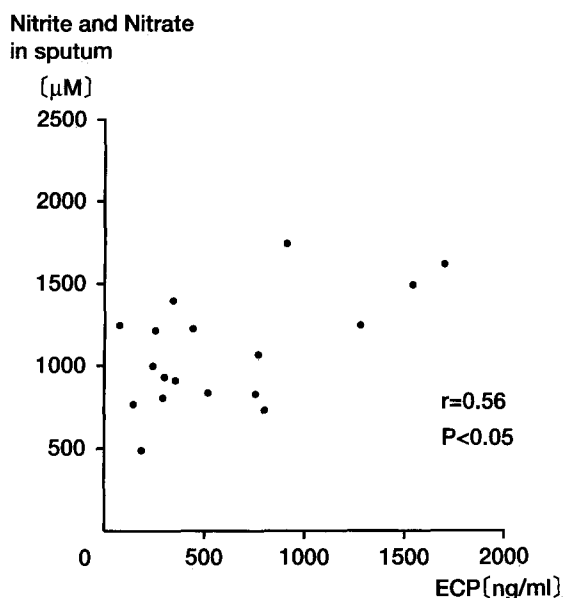


FIG. 3. Relationship between ECP levels and NO levels in induced sputum in patients with asthma.

$115 \mu\text{mol/L}$; $p < 0.05$) (Fig. 1). Percentages of eosinophil counts were significantly higher in patients with asthma (mean, 32.3%) than in normal control subjects (mean, 0.1%; $p < 0.01$). Similarly, ECP levels were also significantly higher in patients with asthma (mean, 608 ng/ml) than in normal control subjects (mean, 98 ng/ml; $p < 0.01$). However, albumin levels in sputum did not differ significantly between the two groups (means: patients with asthma, 1.0 mg/ml; normal control subjects, 0.9 mg/ml). Eosinophil percentages in sputum in patients with asthma were significantly correlated with the concentration of NO derivatives ($r = 0.63$, $p < 0.01$) (Fig. 2). Moreover, ECP levels in sputum in patients with asthma were also significantly correlated with the concentration of NO derivatives ($r = 0.56$, $p < 0.05$) (Fig. 3). Though we did not find a significant correlation between bronchial hyperreactivity to methacholine and the concentration of NO derivatives in sputum, the concentration of NO derivatives in sputum in patients with asthma was significantly correlated with the degree of airflow obstruction ($\text{FEV}_1/\text{forced vital capacity [FVC]}$) ($r = -0.62$, $p < 0.01$) (Fig. 4). We used the percentages of shedding epithelial cells in sputum as a parameter of NO-related cellular toxicity. Percentages of shedding epithelial cells were significantly correlated with the concentration of NO derivatives ($r = 0.61$, $p < 0.01$) (Fig. 5).

DISCUSSION

In this study we found higher levels of NO derivatives in induced sputum in patients with asthma than in normal control subjects. An inducible form of NOS (iNOS) is involved in inflammatory diseases of the airways, such as bronchial asthma, and iNOS generates

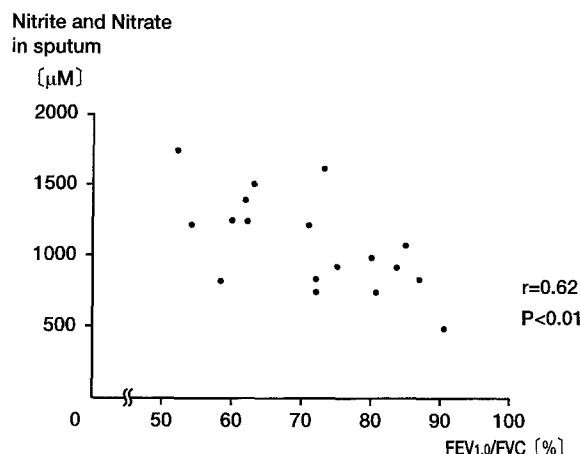


FIG. 4. Relationship between degree of airflow obstruction (FEV₁/FVC) and NO levels in induced sputum in patients with asthma.

much greater amounts of NO than cNOS. The increased levels of NO derivatives in induced sputum in patients with asthma may be due to induction of iNOS by cytokines and other inflammatory mediators released into asthmatic airways. In support of this hypothesis, there is immunocytochemical evidence for iNOS expression in the airway epithelium of patients with asthma but not in that of nonasthmatic individuals.¹² Moreover, NO is detectable in exhaled air of animals and normal human beings,¹³ and its concentration is increased in the exhaled air of patients with asthma.¹⁴ Glucocorticoids prevent the induction of iNOS by cytokines in epithelial cells.¹⁵ Oral prednisolone reduces the level of exhaled NO in patients with asthma but has no effect on the levels of exhaled NO in normal individuals, providing further evidence that the elevated levels of exhaled NO in patients with asthma are due to iNOS expression.¹⁶ Four of the 18 patients with asthma in this study had received inhaled corticosteroids. These patients tended to have lower levels of NO derivatives in induced sputum than patients with asthma who had not received steroids. Recent reports have shown that an increase in the concentration of NO in exhaled air is associated with a decrease in FEV₁ in allergen-induced late asthmatic reactions.¹⁷ In this study we also found that an increased level of NO derivatives in induced sputum was significantly correlated with the degree of airflow limitation (FEV₁/FVC) in patients with asthma. We did not find any correlation between levels of NO derivatives in induced sputum and airway hyperresponsiveness, but most of the patients included in this study had very mild asthma. Further studies are needed to relate the production of endogenous NO to airway hyperresponsiveness, such as measurement of NO in exhaled air and in bronchial biopsy specimens.

The cellular source of NO in induced sputum is unknown. The increased NO in induced sputum in patients with asthma is probably derived from iNOS

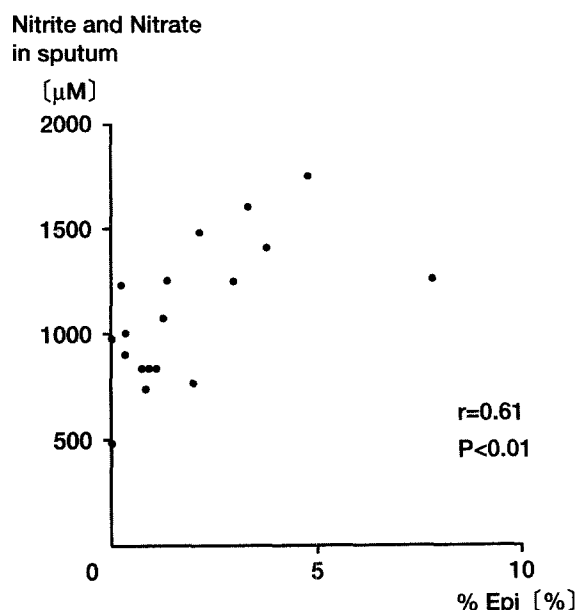


FIG. 5. Relationship between percentages of shedding epithelial cells and NO levels in induced sputum in patients with asthma. Epi, Epithelial cells.

activity in the respiratory tract. Immunohistochemical studies of bronchial biopsy specimens suggest that it may be derived largely from epithelial cells, and there is little evidence for expression of iNOS in airway macrophages.¹⁴ However, the increased NO in induced sputum may be derived not only from epithelial cells but also from infiltrating inflammatory cells. It would be of interest to attempt to determine the distribution of iNOS messenger RNA in the inflammatory cell population of sputum from patients with asthma.

Eosinophilic inflammation in asthma is driven by helper T lymphocytes (T_{H2}), which secrete IL-5. Nitric oxide may amplify allergic inflammation by selective inhibition of T lymphocytes that secrete interferon- γ (T_{H1}), which suppresses the proliferation of T_{H2} cells.¹⁸ This would allow multiplication of T_{H2} lymphocytes, which secrete IL-5. In fact, we found that NO levels in induced sputum were positively correlated with percent eosinophil numbers and the concentration of ECP. The clinical implication of our findings is that measurement of NO derivatives in induced sputum may be useful for assessing allergic inflammation in the airways.

Potentially toxic levels of peroxynitrite can be achieved in tissues under conditions in which NO and superoxide anion production are stimulated. Furthermore, epithelial cells express iNOS after exposure to superoxide anion through activation of the transcription nuclear factor- κB , which is critical for transcription of the iNOS gene.¹⁹ Therefore cytokine-enhanced cellular production of NO and superoxide anion may lead to formation of peroxynitrite in vivo, and peroxynitrite may play an important role in the pathophysiology of airway

injury.²⁰ In this study we found that NO levels in induced sputum were significantly correlated with the percentages of shedding epithelial cells. Nitric oxide generated by iNOS in airway epithelial cells combined avidly with superoxide anion generated by inflammatory cells in the airways to form peroxynitrite, which may have inflammatory effects directly and through the generation of toxic hydroxyl radicals that may contribute to airway epithelial shedding in asthma.²¹ It is possible that the epithelium damaged by accelerated production of endogenous NO is easily shed by artificial stimulants in the presence of airway inflammation. In this study we found that NO may indirectly exacerbate the airway inflammatory response by inducing shedding of epithelial cells, because damage to airway epithelial cells may occur even in patients with relatively mild asthma,²² thereby exposing afferent nerve endings. This might induce the release of sensory neuropeptides through axon reflexes²³ and result in bronchoconstriction, mucus hypersecretion, and microvascular leakage, leading to edema of the airway wall and extravasation of plasma into the airway lumen.

The method used in this study, however, results in underestimation of the absolute effects of NO because of scavenger effects of epithelial lining fluids (ELF).²⁴ It seems likely that the thin layer of ELF may also provide antioxidant production and serve as a front-line defense.²⁵ Because of their location, airway epithelial cells are often exposed to increased intracellular and extracellular concentrations of reactive oxygen species generated by oxidant gases; inflammatory cells such as macrophages, neutrophils, and monocytes; or enzymatic generators such as xanthine oxidase. ELF contains various antioxidant substrates, including vitamin E,²⁶ reduced glutathione,²⁷ and vitamin C.²⁸ In addition, ELF contains a wide spectrum of plasma proteins, some of which, such as ceruloplasmin and transferrin, may function as respiratory tract antioxidants.²⁹ If antioxidant activity in ELF is diminished in certain conditions such as airway inflammation, endogenous NO may have harmful effects on epithelial cells.

However, we believe that NO is one of many mediators that can amplify the airway inflammatory response and that NO and other mediators concomitantly play important roles in airway damage. Moreover, in the method of measuring NO derivatives, there may be no measurable decrease in allergic inflammation end points. Accordingly, it would have been useful to correlate levels of NO derivatives in induced sputum and levels of NO in exhaled air in patients with asthma.

In conclusion, we have found high concentrations of NO derivatives in induced sputum in patients with asthma. It is possible that this increase in levels of NO derivatives may be of pathophysiologic significance in patients with asthma, and the measurement of NO derivatives in induced sputum may also be useful for assessing allergic inflammation.

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